

Thrombin generation

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Theme Issue Article

Thrombin generation, a function test of the haemostatic-thrombotic system

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Summary

By the use of a fluorogenic thrombin substrate and continuous calibration of each individual sample, it is now possible to obtain a thrombin generation (TG) curve (or thrombogram) in plasma, with or without platelets, in an easy routine procedure at high throughput and with an acceptable experimental error (< 5%). Evidence is growing that the parameters of the thrombogram, and notably the area under the curve (endogenous thrombin potential, ETP), are useful in assessing bleeding- or thrombotic risk and its modification by antithrombotic- or haemostatic treatment. Available data strongly suggest that conditions (congenital, acquired, drug-induced) that increase TG all cause a

thrombotic tendency and that conditions that decrease TG prevent thrombosis but, beyond a limit, cause bleeding. Diminution of TG is a common denominator of all antithrombotic treatment, including anti-platelet drugs. The thrombogram can also be used as a tool in the search for new antithrombotics and reflects the haemorrhagic or thrombotic side effects of other drugs (e.g. oral contraceptives). The thrombogram thus is a promising new approach to clinical management of bleeding and thrombotic disease as well as a tool in drug research and epidemiology. Our experience at this moment is insufficient, however, to already clearly define its limits.

Keywords

Thrombin generation, hypercoagulability, antithrombotics, haemophilia

Thromb Haemost 2006; 96: 553–61

Introduction

As we know since Virchow, the occurrence and extent of thrombosis is determined by the force of the local trigger, the local flow conditions and the readiness of the blood to clot. For a long time this “coagulability” has been silently identified with clotting times, as the name already indicates. However, more than 95% of all thrombin forms *after* clotting has taken place (1), and one may well pose the question why (2). Anyhow, it is there and is likely to serve a function. It is therefore potentially interesting to assess this independent variable and investigate its clinical usefulness. Independent variable, indeed, because the time that it takes for clotting to start may or may not relate to the bulk amount of thrombin formed. For example, tissue factor (TF)-induced clotting times correlate with the amount of thrombin formed during anti-vitamin K treatment but do not in heparin treatment, where a modified clotting time (the activated partial thromboplastin time: aPTT) has to be used. Other antithrom-

botics (e.g. dermatan sulphate) do not prolong any clotting time but they do decrease the amount of thrombin formed. Clotting times and the amount of thrombin formed thus are different things and both merit to be estimated. The reason that this has not been done on a large scale was presumably for purely practical reasons—doing a thrombin generation experiment with the methods available in the early 1980s was close to slave labour. Nevertheless, there were laboratories, such as that of Barrow-cliffe in London (3, 4) or Josso in Paris (5, 6), where this approach was never entirely abandoned. Preserving the heritage of Josso – notably his efforts to bring biochemical research to a direct clinical use – we took up TG measurement in the late 1980s. Noting the interesting type of information that could be obtained, on the one hand we applied it to problems such as the mode of action of heparins and the mechanism of platelet procoagulant action, and on the other hand – and to make it simpler – we automated it further and further. Over the last few years this has resulted in a method that allows the thrombogram to be determined

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at high throughput in the clinical laboratory (7) with a quite acceptable experimental error (< 5%). In so far as the present evidence goes, it certainly seems to be a welcome extension of the possibilities of the coagulation laboratory.

It does, indeed, produce information that the clotting time will not give. Excessive thrombin formation, for example, always causes a thrombotic tendency but hardly affects any clotting time. It also yields information that can not be obtained from single factor determinations, because the extraordinary complexity of the thrombin generating mechanism makes means that there is no simple relation between factor concentrations and overall function. It is well known, for example, that although haemophilia is a single-factor-disease, the level of the missing factor is not a reliable indicator of the actual bleeding tendency (8). This is probably because the state of the remainder of the clotting system – including the platelets (9) – determines whether or not a given haemophiliac can make sufficient use of the small residual amount of the missing factor.

The place of thrombin in H&T physiology, or textbook wisdom challenged

How can thrombin be that important? Until yesterday – and until today in most textbooks – the reigning paradigm of haemostasis states that firstly platelets plug the wound and in a second stage thrombin is formed, which cements together this platelet plug; the bleeding time reflects the first phase and the clotting time the second. This view is based on the classical observation that haemophiliacs have a normal bleeding time and a prolonged clotting time, whereas thrombopenia (-pathy) results in a normal clotting time but a prolonged bleeding time. There are – and have always been – observations that challenge this "textbook view". Prolonged bleeding times have, for example, been observed when TG is profoundly affected, as in severe overdosage of oral anticoagulants (10) or heparin (11). Recently it has been found that mice with defective platelet thrombin receptors show a prolonged bleeding time [see further (12)]. The fact that thrombin is formed before a wound stops bleeding is also in agreement with the old observation that products of thrombin action, such as activated factors V and VIII, appear within seconds in the blood flowing from a wound and diminish when heparin is administered (13). The activity of thrombin in such blood must be dependent upon platelet function because it is inhibited by aspirin intake (14–17). Recently, early thrombin formation was most convincingly demonstrated in the excellent experiments by the Furie group [as reviewed in (18)]. They visualised exposed tissue factor, aggregated platelets and fibrin at the site of a micro-trauma in a small vessel in the mouse and saw fibrin appear as early as 10 – 15 seconds (sec) after the lesion. An optimum concentration of tissue factor, as in the Quick time, clots plasma in 12 sec, hence in these experiments thrombin must have appeared almost simultaneously with the platelet aggregate!

It thus appears that from the very beginning, platelets and plasma achieve haemostasis in close cooperation. In fact, many experiments that support the classical separation between platelet function and the clotting system were conducted under conditions where one of the two was absent or inhibited, such as pla-

telets in anticoagulated blood (-plasma) or platelet-free plasma. As soon as platelets and plasmatic coagulation factors are allowed to cooperate, they invariably show early and strong mutual interaction. Thrombin is the most potent platelet activator [see e.g. (12)] and the platelet membrane provides procoagulant phospholipids (19) that allow explosive TG (20). This platelet-clotting interaction, in which circulating tissue factor plays a key role (21), is probably the most important positive feedback mechanism in haemostasis and is responsible for the sudden arrest of blood flow from a wound.

This is not incompatible with the normal bleeding time in haemophiliacs. Early TG is probably triggered by relatively high concentrations of tissue factor (21, 22) and thus may be formed in a factor VIII and IX independent manner. The late bleeding seen in haemophilia may well be related to the enhanced fibrinolysis that ensues from insufficient activation of thrombin activated fibrinolysis inhibitor (TAFI) (see below).

Not only haemostasis but also thrombus growth requires thrombin generation. Thrombus growth is due to a series of ligand- and receptor interactions that have been studied in detail [(23) and references therein]. As soon as experiments are carried out under conditions where thrombin can form, tissue factor and thrombin appear to play an important role, especially at low (venous) and intermediate (large artery) shear rates (12, 21, 24–27). At high shear rates an effect of thrombin inhibition is not seen on initial thrombus formation but becomes obvious when the experiment is prolonged (25). One of the functions of the aggregate must be to serve as a niche in which a sufficient concentration of thrombin can build up and diffuse into the surroundings. Where the concentration of thrombin is high enough, new platelets will be recruited and will be activated so that more thrombin will form [see also (28–31)]. Undoubtedly, several mechanisms of aggregation and plug- or thrombus- growth exist in parallel. The relative importance of thrombin- and collagen-dependent mechanisms has been shown to be influenced by the type of injury to a vessel wall (22). As we will see below, clinical observations suggest that the thrombin dependent mechanism must not be neglected.

Normal TG in platelet poor plasma

To assess the function of the thrombin generating system in a patient, one should be able to measure the concentration of thrombin as a function of time and space in a platelet aggregate at the site of a lesion. At the moment this is technically impossible. A close approximation that is possible is measurement of TG in PRP in the presence of elements of the vessel wall, i.e. tissue factor (TF) and thrombomodulin (TM). Useful partial information can be obtained from observations in platelet poor plasma (PPP).

The general form of any thrombogram is independent of the measuring system and the experimental conditions. It starts with a lag phase, in which only minute amounts of thrombin are formed, after which the full production starts with a sudden burst of thrombin in nanomolar concentrations (Fig. 1). As has been known for a long time, clotting occurs at the start of the explosive burst, i.e. at the end of the lag time (1). The clotting time therefore is a good estimate of the duration of the lag phase and vice versa. During the lag phase, also called the initiation phase (32), hardly

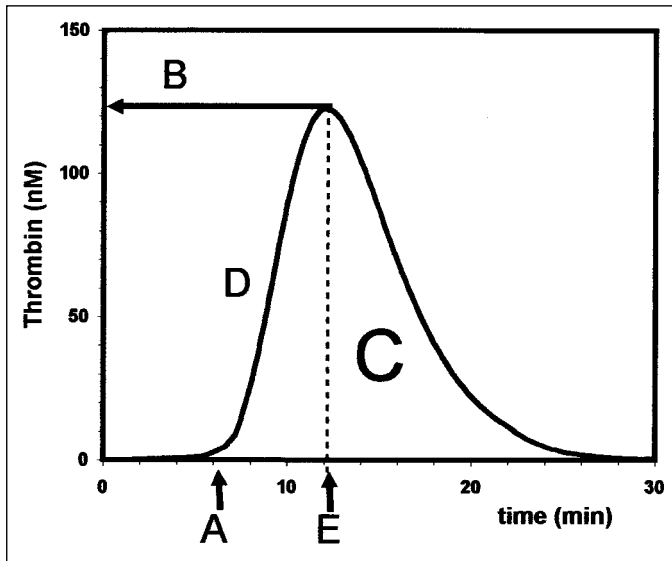


Figure 1: The parameters of the thrombogram. A) Lag time (min), B) Peak height (nM), C) Endogenous thrombin potential (ETP) (= area under the curve)(nM x min), D) maximal rising slope (nM/min), E) time to peak (min).

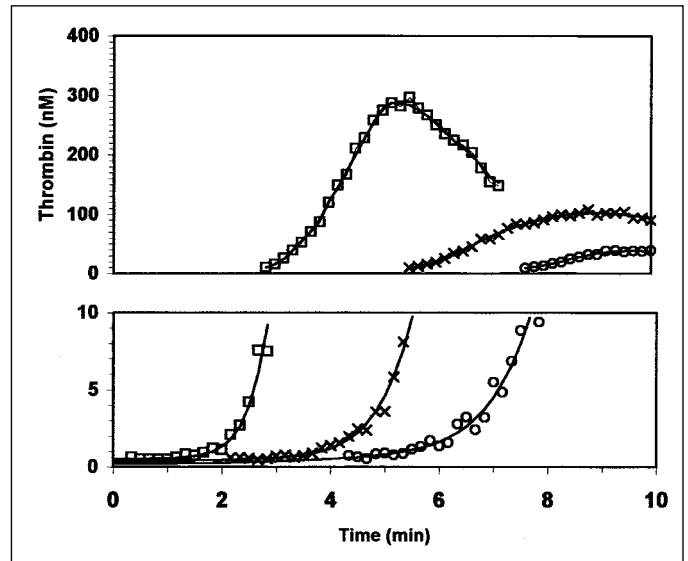


Figure 2: Thrombin generation in contact activated plasma, effect of heparin. (□) Control; (X) 0.02 U/ml unfractionated heparin; (○) 0.03 U/ml unfractionated heparin.

any observable amounts of thrombin form (33) (Fig. 2) which, depending on the reaction conditions, activate one or more of the factors V, VIII, XI and platelets and thus prepare the scene for the full blown TG during the production (also called propagation-) phase (32). The reaction mechanism in the lag phase and the production phase are different. That is the reason why the lag- (=clotting-) time does not automatically contain information on the amount of thrombin formed in the following peak (Fig. 3).

There is no sharp distinction between the production phase and the inactivation phase. As soon as thrombin appears it is scavenged by the plasma antithrombins, even during the lag phase. The velocity of inactivation increases proportionally with the thrombin concentration. At the peak, thrombin generation and decay are equally fast. Once past the peak, decay gains on prothrombin conversion, and somewhere in the down slope the latter will stop. This can be shown by calculating prothrombin conversion velocity from TG and the reaction constant of thrombin – antithrombin interaction (34, 35).

It is common knowledge that the pathway of thrombin formation is dependent upon the type and the amount of trigger used. The physiological trigger is tissue factor (TF). There is, however, no “physiological” concentration of tissue factor because, *in vivo*, it is not a soluble reactant but a component of membranes that are large compared to molecules. Reaction velocities are therefore determined by diffusion rather than by chemical interaction. The same is true for the trigger of the protein C system, thrombomodulin (TM). These two components, TF and TM, need to be added in order to substitute for the (wounded or intact) vessel wall. For practical reasons they are added in soluble form. The concentration of TF is chosen on basis of the section of the reaction mechanism that one wants to study (see below), that of TM so as to produce, in normal plasma, about ~ 50% inhibition of the peak or of the ETP.

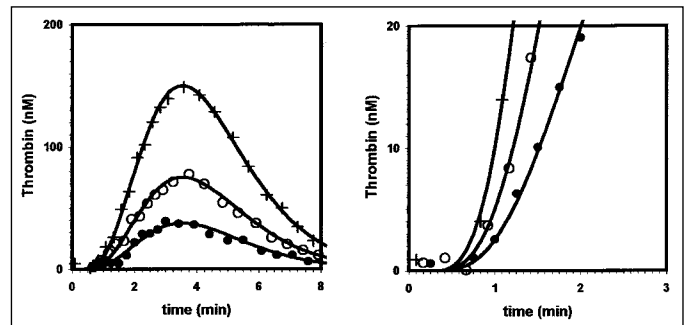


Figure 3: Thrombin generation triggered with 25 pM tissue factor, effect of heparin. (X) Control; (○) 0.02 U/ml unfractionated heparin; (●) 0.03 U/ml unfractionated heparin.

In PPP, in the presence of ~4 μ M procoagulant phospholipids and at high concentrations of TF (>10 pM), the factor VIII, IX and XI -dependent reactions are bypassed. Under the same conditions but at intermediate concentrations (2–5 pM) of TF, TG is dependent upon the concentration of factors VIII and IX, and at yet lower concentrations factor XI will start to play a role (36). The amount of TM that inhibits TG by 50% decreases with decreasing TF concentration. This stands to reason because TM needs to react with thrombin before APC can be formed. At high TF concentration, TG can be so fast that it is over before enough APC can be formed to delimit prothrombinase action. Adding soluble TF and TM is about as close as we can come to the *in-vivo* situation at the moment. However, it remains an artificial construct. TF and TM are present in the same solution here, whereas *in vivo* they are membrane-bound in different compartments, TF in the wound, and TM on the adjacent intact endothelium.

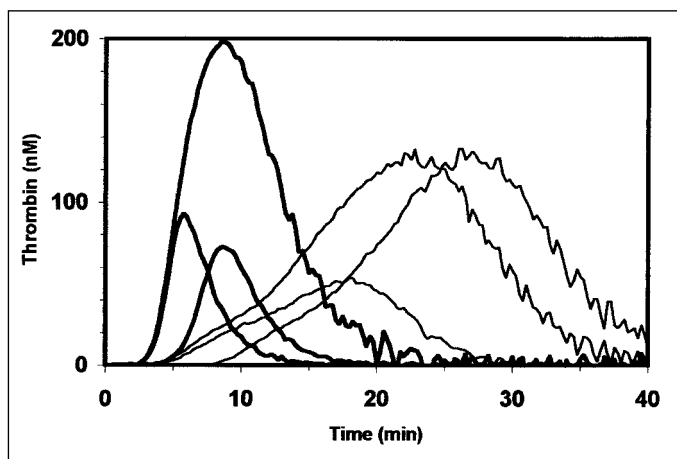


Figure 4: Thrombin generation in PPP and in PRP, effect of rTM (10 nM) and APC (10 nM). Fat lines: PPP, triggered with 5 pM of TF; lean lines PRP, triggered with 0.5 pM of TF. The small peak with the same lag-time as the uninhibited TG is that with added TM. Addition of APC increases the lag-time. Note that platelets appear to induce a resistance to APC but not to TM.

Normal TG in PRP, the roles of fibrin(-ogen)

TG in PPP always requires procoagulant phospholipids to be present. At the phospholipid-solute interface factors Xa and Va adsorb to form prothrombinase (37) and factors IXa and VIIIa to form tenase (38). The surface also serves to “funnel” the substrates (prothrombin and factor X) to these enzyme complexes (39). If TG is observed in PPP without added phospholipids, this is most often due to contamination with platelets or cell remnants during plasma preparation. In carefully prepared plasma it indicated the presence of procoagulant microparticles. In PRP, the platelets provide the procoagulant phospholipids. Platelet activation that is important enough to cause a prolonged rise of intracellular Ca^{++} makes platelets lose the natural asymmetry of their plasma membrane (“scrambling”). Thus, the outside of the platelet enriches in the procoagulant phospholipids phosphatidyl serine (PS) and phosphatidyl ethanolamine (PE) that are normally found at the inside (19). This turns the platelet surface into a two-dimensional reaction compartment, in which adsorbed clotting factors generate thrombin far more efficiently than in free solution (40, 41). Also, procoagulant vesicles are shed from the membrane of such activated platelets. Hence, TG in PRP depends upon platelet activation equally as much as on the plasmatic clotting factors. *In vivo*, there are two other sources of procoagulant phospholipids. These are circulating procoagulant microparticles that tend to crowd on a damaged site (42–44) as well as damaged or apoptotic cells that have lost the normal asymmetry of their membrane (45, 46).

As soon as a fibrin web forms, platelets, but also vesicles, adhere to its fibres (47). This ensures that thrombin is generated on membrane structures that are not able to move in solution. Under these conditions, it is not the chemical reaction velocities, but rather the diffusional transport to and from the membrane structures on the fibres which determine the velocity of TG. As clotting takes place, TG velocity thus shifts from chemical to diffu-

sional control (48–50). When no fibrin can form, chemical kinetics remain in force, so the bulk of thrombin is formed in an essentially different manner in the presence and absence of fibrin. Another effect of the presence of fibrinogen is that it attenuates the formation of α_2 macroglobulin-thrombin (α_2 M-thrombin) (Fig. 5).

Collagen and thrombin are the classical triggers for the platelet procoagulant reaction (19), so a role of the collagen receptors (GPIIb/IIIa, GPVI) and thrombin receptors (PAR 1, 4) was to be expected. Surprisingly, the fibrinogen receptor GPIIb/IIIa was involved (51), not least because it revealed that drugs conceived as aggregation inhibitors, such as abciximab, diminish TG in PRP.

Another unexpected observation was that a clot, as such, provokes procoagulant activity in platelets (52, 53). A snake venom enzyme such as Arvin does not play a role on the platelet or the coagulation system apart from the fact that it clots fibrinogen. Bringing about a clot with Arvin, quite unexpectedly, was found to trigger TG in PRP but not in PPP. The underlying mechanism appeared to be that polymerising fibrin interacts with vWF and modifies it in the same way as adsorption onto collagen or shear stress does. The modified vWF then via GPIb triggers the platelet procoagulant activity (54, 55). In this way fibrin takes over the role of collagen as soon as platelets cover the collagen surface and fibrin forms in the aggregate. Clotting apparently is not the closing act of haemostasis but it is a dynamic part of the haemostatic process. This also explains why thrombin becomes increasingly important the less collagen is exposed (22).

This fibrin-vWF-GPIb mechanism has been verified by showing that TG is diminished in the PRP (but not in the PPP) of hypofibrinogenaemia, in mild von Willebrand disease and in patients with Bernard-Soulier syndrome (in which functional GPIb is congenitally lacking). It reveals a third function of vWF, that not only acts as a “glue” for platelets onto matrix proteins and as a carrier of factor VIII but, in PRP, also contributes to activation of the procoagulant function of platelets and thus can be called a clotting factor in its own right.

TG and thrombosis risk

The pivotal role of thrombin in venous thrombotic disease goes undisputed [e.g. (56)]. This is directly reflected in medical practice: treatment and prevention of venous thrombosis require drugs that diminish thrombin activity in one way or another. This is done by either impeding prothrombin synthesis (vitamin K antagonists), increasing antithrombin activity (heparins), or by inhibiting thrombin directly (hirudin and small-molecular-weight reversible inhibitors). Increased formation of thrombin in PPP always induces a risk of venous thrombosis, whether it is due to deficiency of antithrombin (57), an excess of prothrombin (58), or to any other cause. Disorders in the protein C pathway (deficiency of proteins S and C, factor V_{Leiden}) increase TG as such and becomes particularly obvious if the protein C pathway is activated by thrombomodulin (Fig. 4). The thrombotic tendency induced by oral contraceptives can be explained by the increase of TG caused by an acquired resistance to activated protein C (APC) (59–61) possibly combined with other changes (62, 63).

Particularly interesting is the lupus anticoagulant. This antibody causes an increase of the lag time of thrombin formation, i.e. of the clotting time, and is thus deemed “anticoagulant”. It also brings about an important resistance to the activity of the protein C system and hence an increased TG in the presence of thrombomodulin (64). This again underlines that TG during the lag time is a different process from that in the production phase but that it is the latter that governs the thrombotic tendency. The “LAC paradox”, i.e. prolonged clotting times together with a thrombotic tendency, thus disappears as soon as one resorts to TG.

The role of the plasmatic thrombin generating system is much more evident in venous than in arterial disease. There are arguments to surmise that thrombin plays a part in arterial thrombosis as well (25, 65–67). Excess amounts of factors II, VIII and VII have been found to correlate with the occurrence of myocardial infarction. Also, higher than normal levels of vWF increase TG and are a risk factor for arterial thrombosis (68–74). In a sub-population of young stroke patients (~30%), both TG in PRP and vWF have been shown to be significantly higher than normal (75). Clinical trials have shown that vitamin K antagonists (76) as well as heparins (77, 78) decrease the reoccurrence rate of myocardial infarction. “Antiplatelet” drugs derive at least part of their effect by inhibiting TG in PRP (14, 15, 51). Inversely, aspirin has been shown to be beneficial also in venous thrombosis (79). Like the distinction between primary and secondary haemostasis, the classical view that arterial thrombosis is due to the platelet and venous thrombosis to clotting seems to become less evident. Nevertheless, the precise role of thrombin generation in arterial thrombotic disease remains enigmatic. We surmise that much can be learned from the study of thrombin generation in PRP in relation to arterial disease.

TG and bleeding

It has been demonstrated for the haemophilias (VIII, IX or XI) as well as for all rare clotting factor deficiencies (II, V, VII, X, XII) that TG is diminished and that a clinical bleeding tendency is seen as soon as TG drops below 20% of normal (9, 36, 80, 81). In haemophilia A, not only does infusion of factor VIII or administration of DDAVP augment TG (82), but also inhibitor bypassing therapy with products containing prothrombin and/or factor VII (83–87). Any overshoot of antithrombotic therapy (-prevention) invariably carries a risk of bleeding, because antithrombotics act through diminution of TG.

Severe thrombopenia (<50.000 μl^{-1}) causes decreased TG as well as the Glanzmann (51) and Bernard-Soulier (52) thrombopathies. As mentioned above, in von Willebrand's disease, TG in PRP is significantly impaired. The defect is much more pronounced in PRP than in PPP, which indicates that it can not be explained by the concomitant deficiency of factor VIII. The discrepancy between TG in PPP and in PRP in von Willebrand disease has not yet been explored but is a potentially useful diagnostic possibility.

One of the reasons that a low ETP causes a bleeding tendency is likely due to the fact that the amount of thrombin formed determines the amount of fibrinolysis inhibitor TAFI that is generated (88). Low TG therefore causes increased fibrinolysis. This

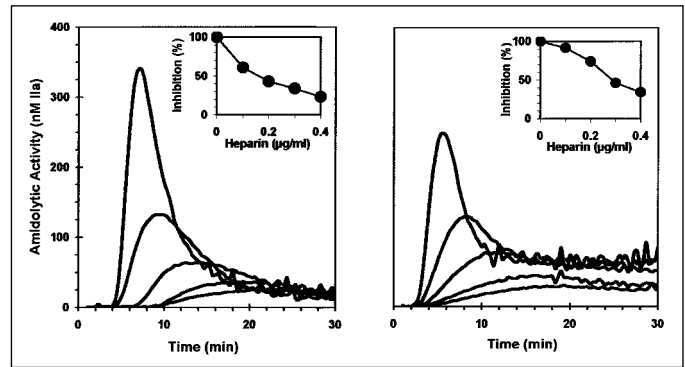


Figure 5: Amidolytic activity in PPP with (left) and without (right) fibrinogen, influence of heparin. Inset: Inhibition of thrombin generation in % as a function of the heparin concentration (0.1, 0.2, 0.3 and 0.4 $\mu\text{g/ml}$).

is likely to be the explanation for the fibrinolytic character of much of the bleeding in haemophilia and would explain the beneficial effect of antifibrinolytic agents [e.g. (89)].

How can the thrombin-forming capacity of blood be assessed?

Classically, thrombograms were obtained by timed subsampling of aliquots from clotting blood or plasma onto a solution of diluted bovine plasma (without Ca^{++}) and by assessing the thrombin concentration in the sample from the clotting time observed (1). The procedure, which takes about one man-hour per curve, involves a score of stopwatches and extraordinary dexterity. Sub-sampling onto a chromogenic thrombin substrate (35) allows automatic recording of the sampling time and semi-automatic measurement of the thrombin activities. In this way, several parallel experiments can be done, which made it possible to recognise the role of various platelet receptors and fibrinogen (51–53).

The use of small MW substrates also introduces a complication because of the exceptional way in which $\alpha_2\text{M}$ macroglobulin ($\alpha_2\text{M}$) inhibits thrombin: After initial proteolytic interaction of thrombin with a “bait”-region, thrombin is bound in a niche of $\alpha_2\text{M}$ so that, through steric hindrance, no physiological substrates can be split anymore. Small substrates can still be attacked, however. The total amidolytic activity measured during TG is the sum of free thrombin and the $\alpha_2\text{M}$ -thrombin complex (Fig. 5). The velocity of the formation of the complex is in good approximation proportional to the concentration of free thrombin. Therefore, the amount of $\alpha_2\text{M}$ -thrombin in serum is proportional to the integral of the thrombogram, i.e. to the ETP. A simple algorithm, executable in any spreadsheet program, allows one to split the curve of amidolytic activity into its free-thrombin and $\alpha_2\text{M}$ -thrombin parts [see further (90)]. In defibrinated plasma, the $\alpha_2\text{M}$ -thrombin end level after TG can be used to estimate the effect of APC. Rosing et al. (60) used this approach to demonstrate the acquired APC-resistance induced by oral contraceptives.

The next step in simplifying TG measurement was to add the chromogenic substrate directly to the clotting plasma. This can

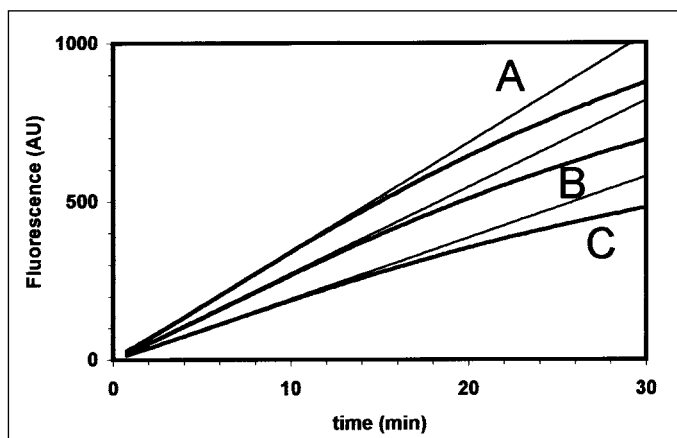


Figure 6: The fluorescence evolving from 100 nM thrombin activity in three different plasmas. The stable thrombin activity was obtained through addition of $\alpha 2M$ -thrombin. A) Normal pooled plasma; B) Haemolytic plasma (0.4 % haemolysis); C) Icteric plasma.

not seriously be attempted with the normal high affinity substrates because they bind thrombin so tightly that they act as potent thrombin inhibitors, so that the normal thrombin generation mechanism, with its many thrombin-driven feedback reactions, will be profoundly disturbed. Once thrombin is formed, the substrate is converted very quickly so the decay phase seen is due to substrate exhaustion and not to thrombin disappearance. The resulting curves have a superficial similarity to TG curves but none of the parameters of the real course of thrombin in time can be read from them.

Correct measurement of the clotting function requires substrates that do not significantly interfere with the normal prothrombin conversion process and have such kinetic properties that, during the whole process, the velocity of product formation is in good approximation proportional to the prevailing concentration of thrombin. Chromogenic substrates of this type are available and allow the monitoring of thrombin activity photometrically (91). Although they do not interfere with normal prothrombin conversion, they will, like any thrombin substrate, compete with the plasmatic antithrombins for the active site of thrombin. Therefore, thrombin decay is always slowed down and the measured ETP is consequently artificially augmented. The magnitude of this effect can be easily calculated and adjusted for [the real ETP equals $K_m/(K_m+S)$ times the observed ETP].

As OD measurement is disturbed by light scattering, clot formation has to be avoided when chromogenic substrates are used, either by defibrination or by adding polymerization inhibitors. Defibrination can be carried out with suitable snake venom enzymes (e.g. Arvin). However, platelets are removed together with the fibrin, so chromogenic continuous methods are restricted to defibrinated PPP. Defibrination abolishes the role of diffusion in TG and significantly changes its kinetics (see above). As discussed, the effect of added heparin, for example, is quite different in normal plasma and defibrinated plasma (Fig. 5). Polymerisation inhibitors may interfere with normal prothrombin conversion and alter the behaviour of platelets (unpublished results).

Fluorescence can be measured in turbid media, so the use of fluorogenic substrates abolishes the drawbacks of defibrination

or inhibition of polymerisation (92). However, it introduces other problems. Firstly, plasma adsorbs a significant and variable amount of the light, so that the signal from plasma, even at 2:5 dilution, is on the mean $\sim 65\%$ of that in buffer. Worse is the large variation of this property between apparently normal plasmas: $\sim 15\%$. This means that the reaction velocities measured in plasma can not be standardised by comparison to buffer, or by comparison to another plasma. Or, if thrombin activity in buffer is used as a standard [as for example in (86, 93, 94)], thrombin generation will be $\sim 30\%$ underestimated and $\sim 15\%$ is added to the experimental variation.

Using the available substrates, the proportionality between reaction velocity and product formation can not be maintained during the experiment and also the fluorescent signal is not proportional to the concentration of fluorophore in the solution. As a result, the same amount of thrombin activity causes a far greater increase of signal at the beginning of the experiment than towards the end. In other words, the calibration factor is a function of the amount of fluorescent product already present. Moreover, the readings are very sensitive to the colour of the plasma, notably to minimal haemolysis (Fig. 6).

The three drawbacks of fluorescent methods have to be dealt with by continuous, individual calibration of every sample. In this technique, a fixed amount of constant thrombin activity is added to a parallel sample of the same plasma. From the resulting curve, the calibration factor at any level of fluorescence is read and the exact thrombin concentration in the sample where TG is taking place can be calculated. Using continuous calibration significantly reduces the intra-individual experimental error from $>15\%$ to $\sim 5\%$.

Methods that use fibrinogen as an indicator substance (95–97) are ideal for the determination of the length of the initiation phase, i.e. the clotting time, especially as no foreign substances have to be added. When the mechanical properties of the clot serve as an indicator, an additional advantage is that whole blood can be used directly (98). Alternatively, the turbidity of the clot in plasma can be measured (99). The greatest drawback of fibrinogen-based methods is that fibrinogen is consumed long before thrombin formation is over so that no information can be obtained on the stages of thrombin generation beyond the very beginning of the production (= propagation) phase. A second drawback is that there is no known relation between mechanical properties or turbidity and the activity of thrombin, so that even the information on this first stage of TG can not be quantified in terms of thrombin concentrations. This is all the more serious when curves are constructed that can easily be mistaken for real thrombograms (100–102) as a result of mathematical transformations that do not improve the content of information in any way. The question remains whether the observed signals provide more useful information than the clotting time does.

It is perhaps now appropriate to remark that a multitude of different methods allow scores of measurable properties of blood (-plasma) that change as a result of the formation of thrombin and/or fibrin. Parameters can be obtained, e.g. with unsuitable substrates, or can be derived from the mechanical properties of forming fibrin (clotting times, thrombelastogram) or from its turbidity (wave form analysis). Other parameters arise when data from suitable substrates are inadequately calibrated etc. Often it

will be possible to correlate such changes to a clinical picture (103). There is a respectable clinical tradition of linking misunderstood phenomena to clinical diagnosis (e.g. spider naevi > liver cirrhosis). This may also extend to laboratory observations. Erythrocyte sedimentation velocity started out as a pregnancy test. One can not deny the use of such correlations. Nevertheless, laboratory variables, in some way resulting from thrombin action, are better abandoned now that correct assessment of this physiological function has become available.

Perspectives

From the above it looks as if the thrombograms might be an overall function test of the haemostatic-thrombotic system. In the present stage of development, the focus is primarily to explore its possibilities. It will inevitably be followed by a phase in which the merits of the test will be critically explored and the limits of its use defined. A few domains in which we expect the test to be useful are mentioned below. Future research will show which of the present projections will be realized.

A: Hypercoagulability and the risk of thrombosis: Following on from the observation that all known risk factors increase TG, one might infer that increased TG is a risk factor as such, independent of its cause. This is reinforced by the observation that TG increases with age in a population without known risk factors (104, 105). The large inter-individual variation of the ETP in the normal population (CV 16%) makes it not uncommon for a normal person to generate twice as much thrombin as any other equally normal person! Larger epidemiological studies seem justified to see whether such differences influence the occurrence of thrombosis. A relation between TG in PPP and venous thrombosis appears quite likely, especially when the function of the protein C system is probed by adding TM. The subject of thrombin generation in PRP and arterial thrombosis currently remains completely unexplored.

B: Control of antithrombotic therapy: At the moment, different tests are being used for the control of different antithrombotics. Diminution of TG is a common denominator of all anticoagulation, including that by low-molecular-weight heparins and heparin likes that hardly influence any clotting test. It also moni-

tors the combined effect of oral anticoagulation and heparins, as in the treatment of venous thrombosis.

The real challenge in this domain, however, is investigating the effect of anticoagulant drugs and platelet inhibitors on TG in PRP. Does platelet inhibition enhance the effect of heparin because it inhibits the release of platelet factor 4? Is the relative APC-resistance of PRP enhanced by oral anticoagulation?; etc. etc. The (clinical-) pharmacology of antithrombotic drugs acquires a new playground now that experiments on the isolated target organ blood are possible.

C: Closely related to the previous item is the role that TG may play in finding and testing of new antithrombotics. We dare to surmise that a candidate drug that inhibits TG by about 40–60% for 24 hours per day, will have an antithrombotic effect and will not cause bleeding. As a screening test TG may significantly reduce the number of experimental animals required to establish the efficacy of new drugs. It may also be useful to monitor a new drug in human experiments and clinical trials. It might even lead to the discovery of drugs that can hardly be found in another way. One might dream, for example, of an orally available heparin-like compound which acts via heparin cofactor II (HCII). Because there is only ~ 1 µM of HCII in plasma, complete activation will never scavenge all the thrombin that can be formed from prothrombin, presently ~2 µM, so overdosage is impossible. Such a drug could not be detected with any clotting test but will immediately show its effect in a TG experiment.

D: In haemophilia the discrepancy between factor VIII level and clinical bleeding tendency remains to be solved. Pilot experiments have shown that TG can be used for therapeutic decisions in inhibitor bypassing therapy (83). If it could be proven that the level of TG – in PPP or in PRP – indeed indicates the bleeding risk, not only would clinical decision making be much easier, but one could also make a more economic use of very costly preparations.

E: Diagnosis of von Willebrand disease: Because vWF is required for the development of full procoagulant power in platelets, a discrepancy between TG in PPP and PRP is systematically found in von Willebrand disease. This could be explored as a means of diagnosis.

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